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(54) Title: A HIGH THROUGHPUT ASSAY USING FUSION PROTEINS

(57) Abstract

This application describes a high throughput assay for screening compounds which are capable of binding to a fusion protein which consists of a target protein and an FK506–binding protein. This application also describes an assay for screening compounds which inhibit a protease.

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TITLE OF THE INVENTION

A HIGH THROUGHPUT ASSAY USING FUSION PROTEINS

BACKGROUND OF THE INVENTION

Several assays have been developed that screen compounds to determine if they are capable of binding to a fusion protein. One such method uses heterobifunctional cross-linking with diamino-europium-cryptate and a protein of interest. However, the activity of the protein is often affected by the modification with the heterobifunctional cross-linking agents. These methods also require an additional purification for the heterogeneous products resulting from the covalent modifications of the proteins.

A new method, Homogeneous Time-Resolved Fluorescence (HTRF), combines the benefits of a fluorescent label and a homogeneous assay protocol in a high throughput screen. HTRF uses a pair of chemically stable labeling molecules, such as a lanthanide cryptate and a fluorescent molecule. Specifically, europium cryptate (Eu(K)) and XL-665 have been used to label biomolecules. XL-665 is a cross-linked derivative of allophycocyanin, a red-green algae pigment. HTRF works by measuring the change in fluorescent energy. If XL-665 is in proximity to Eu(K), it will yield an amplified and long-lived signal. The amount of energy transfer is a function of proximity of the two molecules. Eu(K) has a long-lived fluorescent signal which facilitates the homogeneous nature of the assay. A time delay in reading the signal eliminates the principal difficulty in applying fluorescence to screening formats. This technology is useful in both binding and protease assays. [Kolb et al., J. of Biomolecular Screening, 1, No. 4, 203-210 (1996); Kolb et al., Pharm. Manuf. Int., p. 31 (1996)]

SUMMARY OF THE INVENTION

The instant invention relates to a method of screening that can be used to determine if compounds are capable of binding to a protein or are capable of blocking ligand-protein or protein-protein interactions. The instant invention covers a method of screening for

compounds capable of binding to a fusion protein which comprises combining a test compound, a biotinylated ligand, a fusion protein (target protein, peptide linker and FK506-binding protein), a donor-labeled ligand, and acceptor-labeled streptavidin, and then measuring the fluorescence attributable to the binding of the biotinylated ligand to the fusion protein in the presence of the test compound relative to a control assay in the absence of the test compound, so as to determine the effect the test compound has on the binding of the biotinylated ligand. The instant invention also covers a method of screening for compounds which inhibit a protease which comprises combining a test compound, a fusion protein substrate (reporter protein, peptide linker and FK506-binding protein), a protease, a donor-labeled ligand, and acceptor-labeled streptavidin, and then measuring the fluorescence attributable to the binding of the intact fusion protein substrate in the presence of the test compound relative to a control assay in the absence of the test compound, so as to determine the effect the test compound has on proteolytic activity. This invention provides an immediate means of making use of HTRF technology for the functional assay of either ligand binding to a single or multiple signal transduction domain(s), tyrosine phosphatases, nuclear receptors or bacterial tRNA synthetases in a signal increase assay or proteolytic activity in a signal decrease assay. The present invention is readily adaptable to robotic automation for high capacity screening for agonists, antagonists, and/or inhibitors.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1: Binding of the donor-labeled ligand (Eu(K) labeled ligand for FKBP), fusion protein (FKBP:SH2), biotinylated ligand and the acceptor-labeled streptavidin (SA-XL665), which involves a fluorescent energy transfer that can be measured.

FIGURE 2: Cleaving of donor-labeled ligand (Eu(K) labeled ligand for FKBP), fusion protein substrate (FKBP:SH2:ITAM:B) and the acceptor-labeled streptavidin (SA-XL665).

FIGURE 3: Cleaving of donor-labeled ligand (Eu(K) labeled ligand for FKBP), fusion protein substrate (FKBP:acetyl-CoA:B) and the acceptor-labeled streptavidin (SA-XL665).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of screening for compounds which preferentially bind to a target protein or inhibit a protease.

As depicted in Figure 1, an embodiment of this invention is a method of screening for compounds capable of binding to a fusion protein which comprises the steps of:

a) mixing a test compound, a biotinylated ligand, the fusion protein, a donor-labeled ligand and acceptor-labeled streptavidin;
b) incubating the mixture for an appropriate time;
c) measuring the time-resolved fluorescence attributable to the binding of the biotinylated ligand to the fusion protein in the presence of the test compound; and
d) determining the binding of the biotinylated ligand to the fusion protein in the presence of the test compound relative to a control assay run in the absence of the test compound.

As depicted in Figures 2 and 3, another embodiment of this invention is a method of screening for compounds capable of inhibiting a protease which comprises the steps of:

a) mixing a test compound, a fusion protein substrate, a donor-labeled ligand, acceptor-labeled streptavidin, and a protease;
b) incubating the mixture for an appropriate time;
c) measuring the time-resolved fluorescence attributable to the binding of the intact fusion protein substrate to the acceptor-labeled streptavidin in the presence of the test compound; and

d) determining the binding of the intact fusion protein substrate to the acceptor-labeled streptavidin in the presence of the test compound relative to a control assay run in the absence of the test compound.

For Figures 1-3, the following definitions apply to the terms used therein:

Eu(K) = Europium Cryptate
"506" = FK506 analog
FKBP = FK506 Binding Protein
SH2 = Src homology domain 2
12-mer = phosphotyrosine-containing, 12 peptide
B = biotin
$(SH2)_2$ = tandem SH2 domains of ZAP70
ITAM = bisphospotyrosine-containing, 25 residue peptide
SA = streptavidin

The term "fusion protein" refers to a "target protein" fused to an "FK506-binding protein" (FKBP), the two proteins being separated by a "peptide linker". The term "fusion protein substrate" refers to a "reporter protein" fused to an FKBP, the two proteins being separated by a peptide linker.

A "peptide linker" may consist of a sequence containing from about 1 to about 20 amino acids, which may or may not include the sequence for a protease cleavage site. An example of a peptide linker which is a protease cleavage site is represented by the amino acid sequence GLVPRGS. (SEQ. ID. NO. 1).

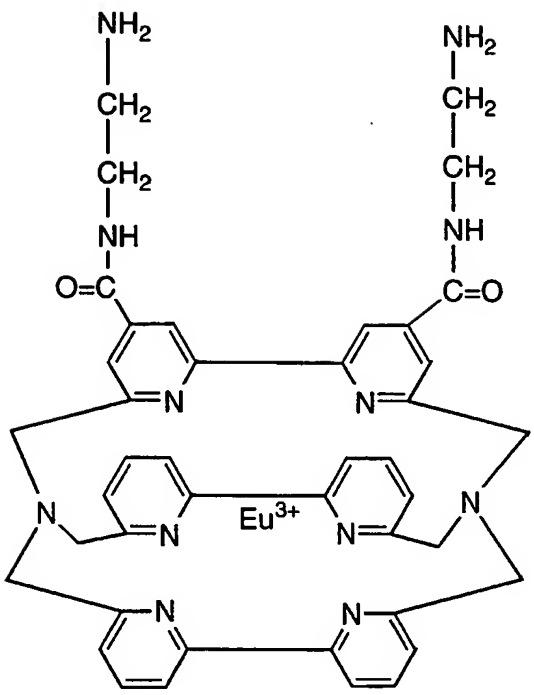
The term "protease" refers to an enzyme that catalyzes the hydrolytic breakdown of proteins and/or peptides. Examples of proteases may include, but are not limited to, thrombin, Human Immunodeficiency Virus (HIV) protease and Tumor Necrosis Factor (TNF) α converting enzyme.

The term "target protein" refers to any protein that has a defined ligand. Types of target proteins include, but are not limited to, tyrosine phosphatases (FKBP-phosphatase chimera as receptor and biotinylated-peptide containing a phosphonomethylene isostere as ligand), nuclear receptors (FKBP-receptor and biotinylated DNA) and bacterial tRNA synthetases (FKBP-tRNA synthetase and biotinylated tRNA). Also included within this definition of target protein are single and multiple signal transduction domains, such as, but not limited to, Src homology 1 (SH1), Src homology 2 (SH2), Src homology 3 (SH3), and pleckstrin homology (PH) domains [Hanks & Hunter, *FASEB J.*, 9, 576-596 (1995); Bolen, *Curr. Opin. Immunol.*, 7, 306-311 (1995); Kuriyan & Cowburn, *Curr. Opin. Struct. Biol.*, 3, 828-837 (1993); Cohen et al., *Cell*, 80, 237-248 (1995)]. The term "SH1 domain" refers to a family of homologous protein domains that bind ATP and catalyze tyrosine phosphorylation of peptide and protein substrates. The term "SH2 domain" refers to a family of homologous protein domains that share the common property of recognizing phosphorylated tyrosine residues in specific peptide contexts. The term "SH3 domain" refers to a family of homologous protein domains that share the common property of recognizing polyproline type II helices. The term "PH domain" refers to a family of homologous protein domains that mediate both protein-protein and protein-lipid interactions. Examples of SH2 domains which may be utilized in the method of the invention include, but are not limited to, the single and tandem SH2 domains present in the tyrosine kinases ZAP70, Syk and Lck. The DNA sequences were obtained from GenBank, National Center for Biotechnology Information, National Library of Medicine, 8600 Rockville Pike, Bethesda, MD 20894. The Accession Numbers for the sequences are: human ZAP70 (L05148) human Syk (L28824) and human Lck (X13529). The sequences for ZAP70, Syk and Lck are disclosed in the sequence listing as follows: the isolated DNA encoding for a fusion protein containing ZAP70 is (SEQ. ID. NO. 2); the isolated DNA encoding for a fusion protein containing Syk is (SEQ. ID. NO. 3); the isolated DNA encoding for a fusion protein containing Lck is (SEQ. ID. NO. 4); the sequence for the

FKBP-ZAP70:SH2 fusion protein is (SEQ. ID. NO. 5); the sequence for the FKBP-Syk:SH2 fusion protein is (SEQ. ID. NO. 6); and the sequence for the FKBP-Lck:SH2 fusion protein is (SEQ. ID. NO. 7).

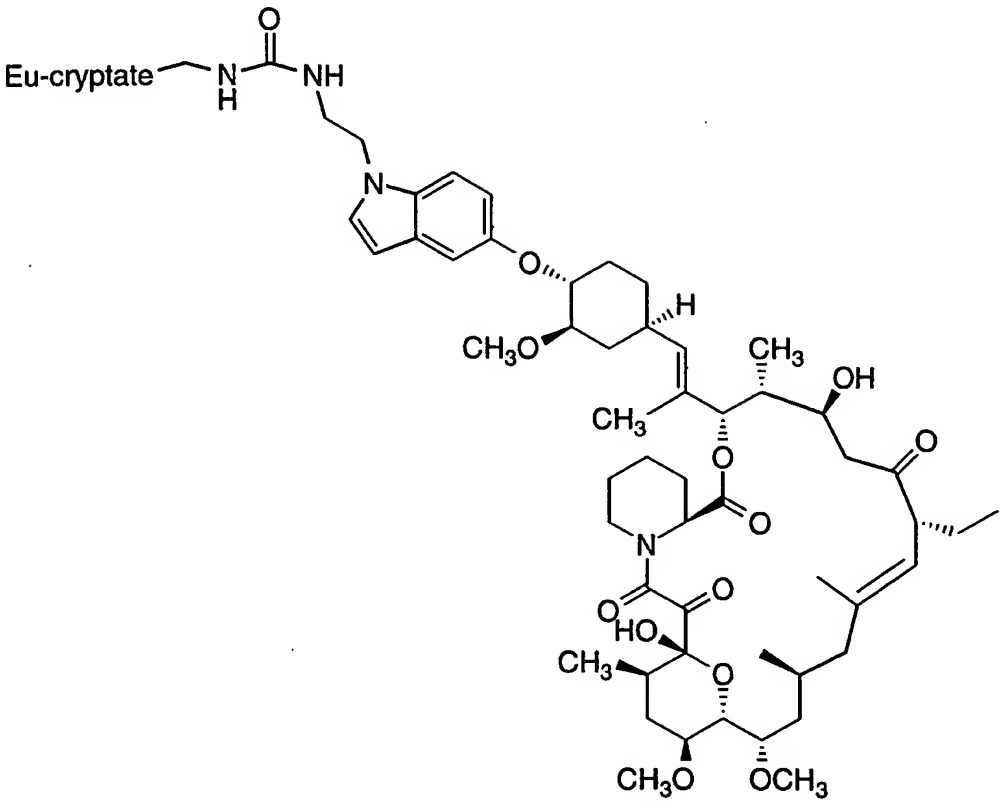
The term "reporter protein" refers to a protein containing covalent biotin or a noncovalently bound biotinylated ligand. Examples of proteins containing covalent biotin are the C-terminal 87 residues of the biotin carboxy carrier protein of acetyl-CoA carboxylase from *Escherichia coli* and the biotin-carrier subunit of transcarboxylase from *Propionibacterium shermannii*. An example of a protein containing a noncovalently bound biotinylated ligand is the tandem SH2 domains of ZAP70 bound to a biotinylated phosphopeptide derived from an Immunoreceptor Tyrosine-based Activation Motif (ITAM) sequence of the human T-cell receptor, the B-cell receptor or a high affinity Immunoglobulin E. (IgE) receptor. Specifically, the Zeta (ζ) 1 sequence of the human T-cell receptor may be utilized. (SEQ. ID. NO. 8) (California Peptide Research Inc., 918 Enterprise Way, Suite 1, Napa, CA 94558)

The term "donor-labeled ligand" refers to an organic cryptate-containing molecule loaded with a fluorescent lanthanide metal which binds to the FKBP. An example of a fluorescent lanthanide metal is europium. The europium-cryptate molecule was obtained from CIS Bio International, Subsidiary of Compagnie ORIS INDUSTRIE SA, Boite Postale 175, F30203 Bagnols sur Ceze Cedex, France, is depicted below.



Europium Cryptate (Eu(K))

An example of a donor-labeled ligand useful in the instant invention is



The term "acceptor-labeled streptavidin" refers to streptavidin coupled to one or more light harvesting molecules. An example of a light harvesting molecule is allophycocyanin and an example of a acceptor-labeled streptavidin useful in the instant invention is SA-XL665. (CIS Bio International, Subsidiary of Compagnie ORIS INDUSTRIE SA, Boite Postale 175, F30203 Bagnols sur Ceze Cedex, France)

The term "control assay" refers to the assay when performed in the presence of the donor-labeled ligand, acceptor-labeled streptavidin, and either fusion protein substrate or fusion protein plus biotinylated ligand, but in the absence of the test compound.

The term FK506-binding proteins may include, but are not limited to, the below listed FKBPs and FKBP homologues, which

include a citation to the references which disclose them. This list is not intended to limit the scope of the invention.

Mammalian	
FKBP-12	Galat et al., *Eur. J. Biochem.*, 216:689-707 (1993).
FKBP-12.6	Wiederrecht, G. and F. Etzkorn *Perspectives in Drug Discovery and Design* , 2:57-84 (1994).
FKBP-13	Galat et al., *supra*; Wiederrecht and Etzkorn, *supra*.
FKBP-25	Galat et al., *supra*; Wiederrecht and Etzkorn, *supra*.
FKBP-38	Wiederrecht and Etzkorn, *supra*.
FKBP-51	Baughman et al., *Mol. Cell. Biol.*, 8, 4395-4402(1995) .
FKBP-52	Galat et al., *supra*.
Bacteria	
Legionella pneumophilia	Galat et al., *supra*.
Legionella micadei	Galat et al., *supra*.
Chlamydia trachomatis	Galat et al., *supra*.
E. coli fkpa	Horne, S.M. and K.D. Young, *Arch. Microbiol.*, 163:357-365 (1995).
E. coli slyD	Roof et al., *J. Biol. Chem.* 269:2902-2910 (1994).
E. coli orf149	Trandinh et al., *FASEB J.* 6:3410-3420 (1992).
Neisseria meningitidis	Hacker, J. and G. Fischer, *Mol. Micro.*, 10:445-456 (1993).
Streptomyces chrysomallus	Hacker and Fischer, *supra*.

Fungal	
yeast FKBP-12	Cardenas et al., *Perspectives in Drug Discovery and Design* , 2:103-126 (1994).
yeast FKBP-13	Cardenas et al., *supra.*
yeast NPR1(FPR3)	Cardenas et al., *supra.*
Neurospora	Galat et al., *supra.*

A variety of host cells used to produce the FKBP chimeras may be used in this invention, which include, but are not limited to, bacteria, yeast, bluegreen algae, plant cells, insect cells and animal cells.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express genes in a variety of host cells, such as, bacteria, yeast, bluegreen algae, plant cells, insect cells and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal cells. An appropriately constructed expression vector may contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses. Commercially available vectors suitable for FKBP fusion protein expression include, but are not limited to pBR322 (Promega), pGEX (Amersham), pT7 (USB), pET (Novagen), pIBI (IBI), pProEX-1 (Gibco/BRL), pBluescript II (Stratagene), pTZ18R and pTZ19R (USB), pSE420 (Invitrogen), pVL1392 (Invitrogen), pBlueBac (Invitrogen), pBAcPAK (Clontech), pHIL (Invitrogen), pYES2

(Invitrogen), pCDNA (Invitrogen), pREP (Invitrogen) or the like. The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, infection, protoplast fusion, and electroporation.

E. coli containing an expression plasmid with the gene for the target or reporter protein fused to FKBP are grown and appropriately induced. The cells are then pelleted and resuspended in a suitable buffer. Although FKBP-12 lacks sequences that specifically direct it to the periplasm, FKBP fusions may be located there and can be released by a standard freeze/thaw treatment of the cell pellet. Following centrifugation, the resulting supernatant contains >80% pure FKBP fusion, which if desired can be purified further by conventional methods. Alternatively, the assay is not dependent on pure protein and the initial periplasmic preparation may be used directly.

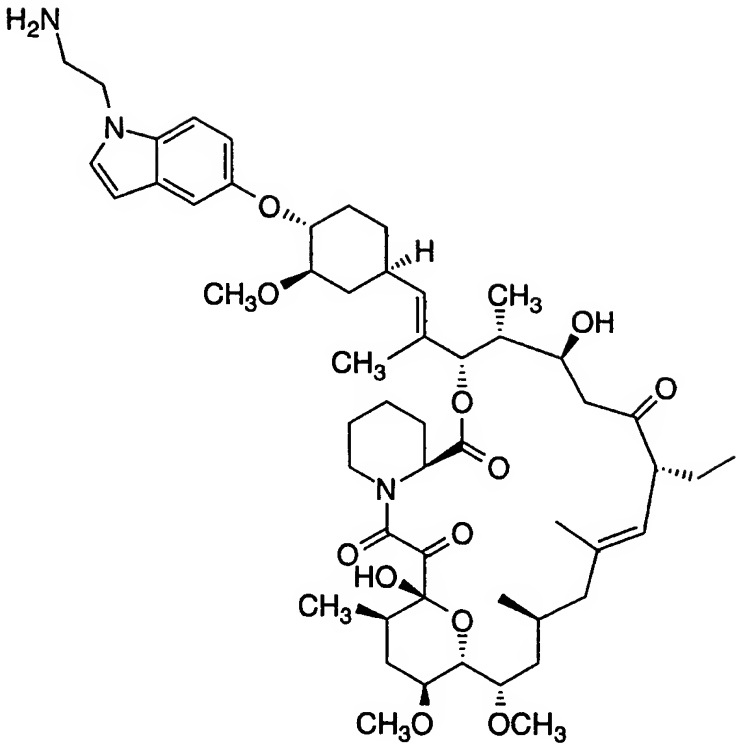
A fusion protein which contains a single or multiple SH2 domain(s) may be purified by preparing an affinity matrix consisting of biotinylated phosphopeptide coupled to avidin or streptavidin immobilized on a solid support. A freeze/thaw extract is prepared from the cells which express the fusion protein and is loaded onto the affinity matrix. The desired fusion protein is then specifically eluted with phenyl phosphate.

To assay the formation of a complex between a target protein and its ligand, the biotinylated ligand is mixed with the FKBP fusion protein in a suitable buffer in the presence of the donor-labeled ligand in the well of a black microplate. After a suitable incubation period to allow complex formation to occur, acceptor-labeled streptavidin is added to capture the tagged ligand and any bound fusion protein. The plate is incubated for a sufficient period to allow the capture to go to completion and then the time resolved fluorescent signal is measured. Screening for agonists/antagonists/inhibitors is carried out by performing the initial incubation prior to the acceptor-labeled streptavidin capture step in the presence of a test compound(s) to determine whether they have an effect upon the binding of the

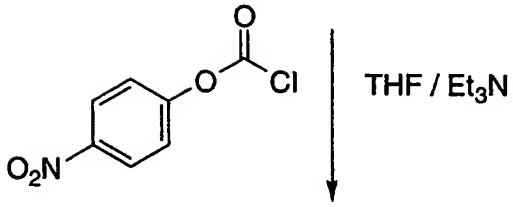
biotinylated ligand to the fusion protein. This principle is illustrated by Figure 1.

To assay the catalytic activity of a protease, a FKBP fusion protein substrate is combined with the protease in a suitable buffer in the presence of the donor-labeled ligand and acceptor-labeled streptavidin in the well of a black microplate. After a suitable incubation period, the time resolved fluorescent signal of the remaining intact fusion protein substrate is measured. Screening for inhibitors is carried out by performing the incubation in the presence of a test compound(s) to determine whether they have an effect upon the cleavage of the fusion protein substrate. This principle is illustrated by Figures 2 and 3.

SCHEME 1



A



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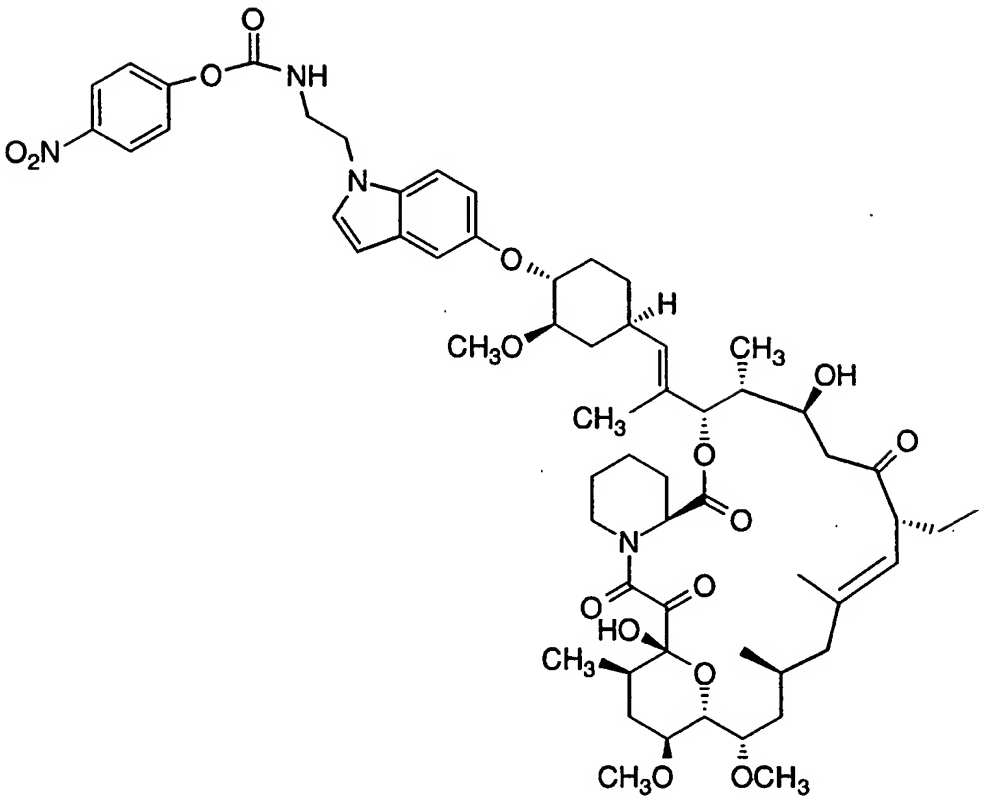
SCHEME 1 (cont'd.)

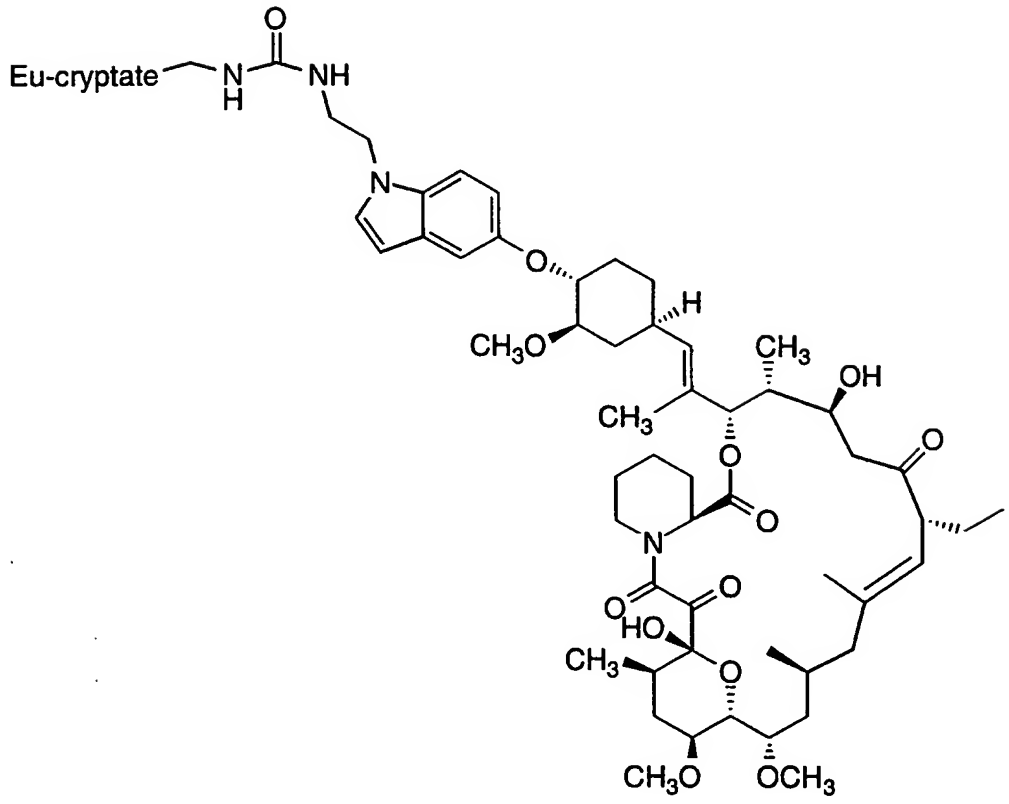


B

Eu-cryptate-NH_2 | THF / Et_3N

5

SCHEME 1 (cont'd.)



C

Scheme 1 depicts the treatment of Compound A, an analog of FK506, with 1 equivalent of p-nitrophenylchloroformate in THF in the presence of a tertiary amine, which gives the p-nitrophenylcarbamate, Compound B. The nitrophenylcarbamate is isolated by silica gel chromatography or used without further purification. The nitrophenylcarbamate is an activated acylating group and will undergo attack by a variety of nucleophiles. The cryptate has two primary amino groups (nucleophiles). Treatment of the p-nitrophenylcarbamate with the europium cryptate in a non-protic

solvent (such as THF) in the presence of a tertiary amine should afford the corresponding urea. Compound C, the cryptate hybrid of the FK506 analog, will be purified by either normal phase silica gel chromatography on prep TLC plates or by reverse phase HPLC chromatography.

The cryptate can be linked to Compound A, other FK506 analogs, and related molecules by a variety of linkages, such as carbamates, amides, ureas, amines, etc. One of ordinary skill in the art would be familiar with the standard procedures used to prepare the FK506 analog containing the cryptate molecule.

The present invention can be understood further by the following examples, which do not constitute a limitation of the invention.

EXAMPLE 1

Process for Preparing the FKBP fusion cloning vector

General techniques for modifying and expressing genes in various host cells can be found in Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. Current Protocols in Molecular Biology (John Wiley & Sons, New York, New York, 1989). Sequences for a 3'- altered FKBP fragment that contained a glycine codon (GGT) in place of the stop (TGA) codon followed by a sequence encoding a thrombin site (Leu-Val-Pro-Arg) and *Bam*HI restriction site (GGATCC) were amplified using the polymerase chain reaction (PCR). The PCR reaction contained the following primers: 5'-GATCGCCATGGGAGTGCAGGTGGAAACCATCTCCCCA-3' (SEQ. ID. NO. 9) and 5'-TACGAATTCTGGCGTGGATCCAC GCGGAACCAGACCTTCCAGTTTTAG-3' (SEQ. ID. NO. 10) and a plasmid containing human FKBP-12 as the template. The resulting 367 base pair amplification product was ligated into the vector pCRII (Invitrogen) and the ligation mixture transformed into competent *Escherichia coli* cells. Clones containing an insert were identified using PCR with flanking vector primers. Dideoxy DNA sequencing confirmed the nucleotide sequence of one positive isolate. The altered

338 base pair FKBP fragment was excised from the pCRII plasmid using *Nco*I and *BamH*I and ligated into *Nco*I and*Bam*HI digested pET9d (Novagen) plasmid. Competent *E. coli* were transformed with the ligation mixture, and colonies containing the insert were identified using PCR with primers encoding for flanking vector sequences. The FKBP fusion cloning vector is called pET9dFKBPt.

EXAMPLE 2

Process for Preparing the FK-ZAP70 fusion expression vector

A DNA fragment encoding for the tandem SH2 domains of ZAP70 was prepared by PCR to contain a *Bam*HI site at the 5'-end such that the reading frame was conserved with that of FKBP in the fusion vector. At the 3'-end, the fragment also incorporated a stop codon followed by a *Bam*HI site. The PCR reaction contained Molt-4 cDNA (Clontech) and the following primers: 5'-ATTAGGATCCATGCCAGATCCTGCAGCTCACCTGCCCT-3' (SEQ. ID. NO. 11) and 5'-ATATGGATCCTTACCAGAGGCGTTGCT-3' (SEQ. ID. NO. 12). The fragment was cloned into a suitable vector, sequenced, digested with *Bam*HI, and the insert containing the SH2 domains ligated to *Bam*HI treated pET9dFKBPt, and transformed into *E. coli*. Clones containing inserts in the correct orientation were identified by PCR or restriction enzyme analysis. Plasmid DNA was prepared and used to transform BL21(DE3) cells.

EXAMPLE 3

Process for Preparing the FK-Syk fusion expression vector

The expression vector for the tandem SH2 domains of Syk fused to FKBP was prepared as in Example 2 except that the PCR reaction contained Raji cell cDNA (Clontech) and the following primers: 5'-CAATAGGATCCATGGCCAGCAGCGGCATGGCTGA-3' (SEQ. ID. NO. 13) and 5'-GACCTAGGATCCCTAATTAACATTTCCCTGTGTGCCGAT-3' (SEQ. ID. NO. 14).

EXAMPLE 4

Process for Preparing the FK-Lck fusion expression vector

The expression vector for the SH2 domain of Lck fused to FKBP was prepared as in Example 2 except that the PCR reaction contained Molt-4 cDNA (Clontech) and the following primers:

5'-ATATGGATCCATGGCGAACAGCCTGGAGCCCGAACCCT-3' (SEQ. ID. NO. 15) and

5'-ATTAGGATCCTTAGGTCTGGCAGGGGCGGCTCAACCGTGT GCA-3' (SEQ. ID. NO. 16).

EXAMPLE 5

FK-ZAP70

Step A: Process for Expression of FK-ZAP70

E. coli BL21(DE3) cells containing the pET9dFKBPt/ ZAP70SH2 plasmid were grown in Luria-Bertani (LB) media containing 50 microgram/ml kanamycin at 37 degrees C until the optical density measured at 600 nm was 0.5-1.0. Expression of the FK-ZAP70 fusion protein was induced with 0.1 mM isopropyl beta-thiogalactopyranoside and the cells were grown for another 3-5 hr at 30 degrees C. They were pelleted at 4400 x g for 10 min at 4 degrees C and resuspended in 2% of the original culture volume with 100 mM tris pH 8.0 containing 1 microgram/ml each aprotinin, pepstatin, leupeptin, and bestatin. The resuspended pellet was frozen at -20 degrees C until further purification.

Step B: Process for Purification of FK-ZAP70

The affinity matrix for purification of FK-ZAP70 was prepared by combining agarose-immobilized avidin with excess biotinylated phosphopeptide derived from the ζ1 ITAM sequence of the human T-cell receptor, biotinyl-GSNQLpYNELNLGRREEpYDVLDK, (SEQ. ID. NO. 8) and washing out unbound peptide. Frozen cells containing FK-ZAP70 were thawed in warm water, refrozen on dry ice

for about 25 min., then thawed again. After the addition of 0.1% octyl glucoside, 1 mM dithiothreitol (DTT) and 500 mM NaCl, the extract was centrifuged at 35,000 x g for approximately 30 minutes. The supernatant was loaded onto the phosphopeptide affinity column, at about 4° and washed with phosphate buffered saline containing 1 mM DTT and 0.1% octyl glucoside. FK-ZAP70 was eluted with 200 mM phenyl phosphate in the same buffer at about 37°. The protein pool was concentrated and the phenyl phosphate removed on a desalting column. The purified FK-ZAP70 was stored at about -30° in 10 mM HEPES/150 mM NaCl/1 mM DTT/0.1 mM EDTA/10% glycerol.

EXAMPLE 6

FK-Syk

E. coli BL21(DE3) cells containing the pET9dFKBPt/SykSH2 plasmid were grown, induced, and harvested as described in Example 5. FK-Syk was purified using the same affinity matrix and methodology described in Example 5.

EXAMPLE 7

FK-Lck

E. coli BL21(DE3) cells containing the pET9dFKBPt/LckSH2 plasmid were grown, induced, and harvested as described in Example 5. The affinity matrix for purification of FK-Lck was prepared by combining agarose-immobilized avidin with excess biotinyl-EPQpYEEIPIYL, (SEQ. ID. NO. 17) and washing out unbound peptide. The remaining methodology for purification was the same as Example 5.

EXAMPLE 8

Method of Screening for Inhibitors of FK-ZAP

A DMSO solution of test compound(s) and biotinyl-phosphopeptide stock solution in a suitable buffer are dispensed into the

wells of a 96-well black microplate. Next, a mixture of FK-ZAP protein and Eu(K)-labeled FK506 analog are added to each test well. Finally, a solution of SA-XL665 is dispensed to each well and the plate is incubated for an appropriate time. The fluorescence ratio is then measured in a Packard Discovery HTRF analyzer. (Packard Instrument Company, 800 Research Parkway, Meridan, CT 06450)

EXAMPLE 9

Method of Screening for Inhibitors of Thrombin

A DMSO solution of test compound(s) and a suitable buffer containing a mixture of FK-ZAP protein, Eu(K)-labeled FK506 analog, and biotinyl-phosphopeptide are dispensed into the wells of a 96-well black microplate. Next, thrombin is added to each test well. After an appropriate incubation period, a solution of SA-XL665 is dispensed to each well. The fluorescence ratio is then measured in a Packard Discovery HTRF analyzer. (Packard Instrument Company, 800 Research Parkway, Meridan, CT 06450)

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: HERMES, JEFFREY D.
SALOWE, SCOTT P.
SINCLAIR, PETER J.

(ii) TITLE OF THE INVENTION: A HIGH THROUGHPUT ASSAY USING FUSION PROTEINS

(iii) NUMBER OF SEQUENCES: 17

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: Rahway
(D) STATE: NJ
(E) COUNTRY: USA
(F) ZIP: 07065-0900

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/040,795
(B) FILING DATE: 14 March 1997

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Leu Val Pro Arg Gly Ser
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1137 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGGGAGTGC AGGTGGAAAC CATCTCCCCA GGAGATGGAC GCACCTTCCC CAAGCGCGGC 60
CAGACCTGCG TGGTGCACTA CACCGGGATG CTTGAAGATG GAAAGAAATT TGATTCCTCC 120
CGGGACAGAA ACAAGCCCTT TAAGTTTATG CTAGGCAAGC AGGAGGTGAT CCGAGGCTGG 180
GAAGAAGGGG TTGCCCAGAT GAGTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT 240
TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCCAC CACATGCCAC TCTCGTCTTC 300
GATGTGGAGC TTCTAAAACT GGAAGGTCTG GTTCCGCGTG GATCCATGCC AGATCCTGCA 360
GCTCACCTGC CCTTCTTCTA CGGCAGCATC TCGCGTGCCG AGGCCGAGGA GCACCTGAAG 420
CTGGCGGGCA TGGCGGACGG GCTCTTCCTG CTGCGCCAGT GCCTGCGCTC GCTGGGCGGC 480
TATGTGCTGT CGCTCGTGCA CGATGTGCGC TTCCACCACT TTCCCATCGA GCGCCAGCTC 540
AACGGCACCT ACGCCATTGC CGGCGGCAAA GCGCACTGTG GACCGGCAGA GCTCTGCGAG 600
TTCTACTCGC GCGACCCCGA CGGGCTGCCC TGCAACCTGC GCAAGCCGTG CAACCGGCCG 660
TCGGGCCTCG AGCCGCAGCC GGGGGTCTTC GACTGCCTGC GAGACGCCAT GGTGCGTGAC 720
TACGTGCGCC AGACGTGGAA GCTGGAGGGC GAGGCCCTGG AGCAGGCCAT CATCAGCCAG 780
GCCCCGCAGG TGGAGAAGCT CATTGCTACG ACGGCCCACG AGCGGATGCC CTGGTACCAC 840
AGCAGCCTGA CGCGTGAGGA GGCCGAGCGT AAACTTTACT CTGGGGCGCA GACCGACGGC 900
AAGTTCCTGC TGAGGCCGCG GAAGGAGCAG GGCACATACG CCCTGTCCCT CATCTATGGG 960
AAGACGGTGT ACCACTACCT CATCAGCCAA GACAAGGCGG GCAAGTACTG CATTCCCGAG 1020
GGCACCAAGT TTGACACGCT CTGGCAGCTG GTGGAGTATC TGAAGCTGAA GGCGGACGGG 1080
CTCATCTACT GCCTGAAGGA GGCCTGCCCC AACAGCAGTG CCAGCAACGC CTCTTAA 1137

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1155 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGGGAGTGC AGGTGGAAAC CATCTCCCCA GGAGATGGAC GCACCTTCCC CAAGCGCGGC 60
CAGACCTGCG TGGTGCACTA CACCGGGATG CTTGAAGATG GAAAGAAATT TGATTCCTCC 120
CGGGACAGAA ACAAGCCCTT TAAGTTTATG CTAGGCAAGC AGGAGGTGAT CCGAGGCTGG 180
GAAGAAGGGG TTGCCCAGAT GAGTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT 240
TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCCAC CACATGCCAC TCTCGTCTTC 300
GATGTGGAGC TTCTAAAACT GGAAGGTCTG GTTCCGCGTG GATCCATGGC CAGCAGCGGC 360

```
ATGGCTGACA GCGCCAACCA CCTGCCCTTC TTTTTCGGCA ACATCACCCG GGAGGAGGCA  420
GAAGATTACC TGGTCCAGGG GGGCATGAGT GATGGGCTTT ATTTGCTGCG CCAGAGCCGC  480
AACTACCTGG GTGGCTTCGC CCTGTCCGTG GCCCACGGGA GGAAGGCACA CCACTACACC  540
ATCGAGCGGG AGCTGAATGG CACCTACGCC ATCGCCGGTG GCAGGACCCA TGCCAGCCCC  600
GCCGACCTCT GCCACTACCA CTCCCAGGAG TCTGATGGCC TGGTCTGCCT CCTCAAGAAG  660
CCCTTCAACC GGCCCCAAGG GGTGCAGCCC AAGACTGGGC CCTTTGAGGA TTTGAAGGAA  720
AACCTCATCA GGGAATATGT GAAGCAGACA TGGAACCTGC AGGGTCAGGC TCTGGAGCAG  780
GCCATCATCA GTCAGAAGCC TCAGCTGGAG AAGCTGATCG CTACCACAGC CCATGAAAAA  840
ATGCCTTGGT TCCATGGAAA AATCTCTCGG GAAGAATCTG AGCAAATTGT CCTGATAGGA  900
TCAAAGACAA ATGGAAAGTT CCTGATCCGA GCCAGAGACA ACAACGGCTC CTACGCCCTG  960
TGCCTGCTGC ACGAAGGGAA GGTGCTGCAC TATCGCATCG ACAAAGACAA GACAGGGAAG 1020
CTCTCCATCC CCGAGGGAAA GAAGTTCGAC ACGCTCTGGC AGCTAGTCGA GCATTATTCT 1080
TATAAAGCAG ATGGTTTGTT AAGAGTTCTT ACTGTCCCAT GTCAAAAAAT CGGCACACAG 1140
GGAAATGTTA ATTAG                                                   1155
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 675 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
ATGGGAGTGC AGGTGGAAAC CATCTCCCCA GGAGATGGAC GCACCTTCCC CAAGCGCGGC   60
CAGACCTGCG TGGTGCACTA CACCGGGATG CTTGAAGATG GAAAGAAATT TGATTCCTCC  120
CGGGACAGAA ACAAGCCCTT TAAGTTTATG CTAGGCAAGC AGGAGGTGAT CCGAGGCTGG  180
GAAGAAGGGG TTGCCCAGAT GAGTGTGGGT CAGAGAGCCA AACTGACTAT ATCTCCAGAT  240
TATGCCTATG GTGCCACTGG GCACCCAGGC ATCATCCCAC CACATGCCAC TCTCGTCTTC  300
GATGTGGAGC TTCTAAAACT GGAAGGTCTG GTTCCGCGTG GATCCATGGC GAACAGCCTG  360
GAGCCCGAAC CCTGGTTCTT CAAGAACCTG AGCCGCAAGG ACGCGGAGCG GCAGCTCCTG  420
GCGCCCGGGA ACACTCACGG CTCCTTCCTC ATCCGGGAGA GCGAGAGCAC CGCGGGATCG  480
TTTTCACTGT CGGTCCGGGA CTTCGACCAG AACCAGGGAG AGGTGGTGAA ACATTACAAG  540
ATCCGTAATC TGGACAACGG TGGCTTCTAC ATCTCCCCTC GAATCACTTT TCCCGGCCTG  600
CATGAACTGG TCCGCCATTA CACCAATGCT TCAGATGGGC TGTGCACACG GTTGAGCCGC  660
CCCTGCCAGA CCTAA                                                   675
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 378 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Gly Val Gln Val Glu Thr Ile Ser Pro Gly Asp Gly Arg Thr Phe
 1               5                  10                  15
Pro Lys Arg Gly Gln Thr Cys Val Val His Tyr Thr Gly Met Leu Glu
            20                  25                  30
```

```
Asp Gly Lys Lys Phe Asp Ser Ser Arg Asp Arg Asn Lys Pro Phe Lys
        35                  40                  45
Phe Met Leu Gly Lys Gln Glu Val Ile Arg Gly Trp Glu Glu Gly Val
    50                  55                  60
Ala Gln Met Ser Val Gly Gln Arg Ala Lys Leu Thr Ile Ser Pro Asp
65                  70                  75                  80
Tyr Ala Tyr Gly Ala Thr Gly His Pro Gly Ile Ile Pro Pro His Ala
                85                  90                  95
Thr Leu Val Phe Asp Val Glu Leu Leu Lys Leu Glu Gly Leu Val Pro
            100                 105                 110
Arg Gly Ser Met Pro Asp Pro Ala Ala His Leu Pro Phe Phe Tyr Gly
        115                 120                 125
Ser Ile Ser Arg Ala Glu Ala Glu Glu His Leu Lys Leu Ala Gly Met
    130                 135                 140
Ala Asp Gly Leu Phe Leu Leu Arg Gln Cys Leu Arg Ser Leu Gly Gly
145                 150                 155                 160
Tyr Val Leu Ser Leu Val His Asp Val Arg Phe His His Phe Pro Ile
                165                 170                 175
Glu Arg Gln Leu Asn Gly Thr Tyr Ala Ile Ala Gly Gly Lys Ala His
            180                 185                 190
Cys Gly Pro Ala Glu Leu Cys Glu Phe Tyr Ser Arg Asp Pro Asp Gly
        195                 200                 205
Leu Pro Cys Asn Leu Arg Lys Pro Cys Asn Arg Pro Ser Gly Leu Glu
    210                 215                 220
Pro Gln Pro Gly Val Phe Asp Cys Leu Arg Asp Ala Met Val Arg Asp
225                 230                 235                 240
Tyr Val Arg Gln Thr Trp Lys Leu Glu Gly Glu Ala Leu Glu Gln Ala
                245                 250                 255
Ile Ile Ser Gln Ala Pro Gln Val Glu Lys Leu Ile Ala Thr Thr Ala
            260                 265                 270
His Glu Arg Met Pro Trp Tyr His Ser Ser Leu Thr Arg Glu Glu Ala
        275                 280                 285
Glu Arg Lys Leu Tyr Ser Gly Ala Gln Thr Asp Gly Lys Phe Leu Leu
    290                 295                 300
Arg Pro Arg Lys Glu Gln Gly Thr Tyr Ala Leu Ser Leu Ile Tyr Gly
305                 310                 315                 320
Lys Thr Val Tyr His Tyr Leu Ile Ser Gln Asp Lys Ala Gly Lys Tyr
                325                 330                 335
Cys Ile Pro Glu Gly Thr Lys Phe Asp Thr Leu Trp Gln Leu Val Glu
            340                 345                 350
Tyr Leu Lys Leu Lys Ala Asp Gly Leu Ile Tyr Cys Leu Lys Glu Ala
        355                 360                 365
Cys Pro Asn Ser Ser Ala Ser Asn Ala Ser
    370                 375
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 384 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Gly Val Gln Val Glu Thr Ile Ser Pro Gly Asp Gly Arg Thr Phe
 1               5                  10                  15
Pro Lys Arg Gly Gln Thr Cys Val Val His Tyr Thr Gly Met Leu Glu
            20                  25                  30
Asp Gly Lys Lys Phe Asp Ser Ser Arg Asp Arg Asn Lys Pro Phe Lys
        35                  40                  45
Phe Met Leu Gly Lys Gln Glu Val Ile Arg Gly Trp Glu Glu Gly Val
    50                  55                  60
Ala Gln Met Ser Val Gly Gln Arg Ala Lys Leu Thr Ile Ser Pro Asp
65                  70                  75                  80
Tyr Ala Tyr Gly Ala Thr Gly His Pro Gly Ile Ile Pro Pro His Ala
                85                  90                  95
Thr Leu Val Phe Asp Val Glu Leu Leu Lys Leu Glu Gly Leu Val Pro
            100                 105                 110
Arg Gly Ser Met Ala Ser Ser Gly Met Ala Asp Ser Ala Asn His Leu
        115                 120                 125
Pro Phe Phe Phe Gly Asn Ile Thr Arg Glu Glu Ala Glu Asp Tyr Leu
    130                 135                 140
Val Gln Gly Gly Met Ser Asp Gly Leu Tyr Leu Leu Arg Gln Ser Arg
145                 150                 155                 160
Asn Tyr Leu Gly Gly Phe Ala Leu Ser Val Ala His Gly Arg Lys Ala
                165                 170                 175
His His Tyr Thr Ile Glu Arg Glu Leu Asn Gly Thr Tyr Ala Ile Ala
            180                 185                 190
Gly Gly Arg Thr His Ala Ser Pro Ala Asp Leu Cys His Tyr His Ser
        195                 200                 205
Gln Glu Ser Asp Gly Leu Val Cys Leu Leu Lys Lys Pro Phe Asn Arg
    210                 215                 220
Pro Gln Gly Val Gln Pro Lys Thr Gly Pro Phe Glu Asp Leu Lys Glu
225                 230                 235                 240
Asn Leu Ile Arg Glu Tyr Val Lys Gln Thr Trp Asn Leu Gln Gly Gln
                245                 250                 255
Ala Leu Glu Gln Ala Ile Ile Ser Gln Lys Pro Gln Leu Glu Lys Leu
            260                 265                 270
Ile Ala Thr Thr Ala His Glu Lys Met Pro Trp Phe His Gly Lys Ile
        275                 280                 285
Ser Arg Glu Glu Ser Glu Gln Ile Val Leu Ile Gly Ser Lys Thr Asn
    290                 295                 300
Gly Lys Phe Leu Ile Arg Ala Arg Asp Asn Asn Gly Ser Tyr Ala Leu
305                 310                 315                 320
Cys Leu Leu His Glu Gly Lys Val Leu His Tyr Arg Ile Asp Lys Asp
                325                 330                 335
Lys Thr Gly Lys Leu Ser Ile Pro Glu Gly Lys Lys Phe Asp Thr Leu
            340                 345                 350
Trp Gln Leu Val Glu His Tyr Ser Tyr Lys Ala Asp Gly Leu Leu Arg
        355                 360                 365
Val Leu Thr Val Pro Cys Gln Lys Ile Gly Thr Gln Gly Asn Val Asn
    370                 375                 380
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 224 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Met Gly Val Gln Val Glu Thr Ile Ser Pro Gly Asp Gly Arg Thr Phe
 1               5                  10                  15
Pro Lys Arg Gly Gln Thr Cys Val Val His Tyr Thr Gly Met Leu Glu
            20                  25                  30
Asp Gly Lys Lys Phe Asp Ser Ser Arg Asp Arg Asn Lys Pro Phe Lys
        35                  40                  45
Phe Met Leu Gly Lys Gln Glu Val Ile Arg Gly Trp Glu Glu Gly Val
    50                  55                  60
Ala Gln Met Ser Val Gly Gln Arg Ala Lys Leu Thr Ile Ser Pro Asp
65                  70                  75                  80
Tyr Ala Tyr Gly Ala Thr Gly His Pro Gly Ile Ile Pro Pro His Ala
                85                  90                  95
Thr Leu Val Phe Asp Val Glu Leu Leu Lys Leu Glu Gly Leu Val Pro
            100                 105                 110
Arg Gly Ser Met Ala Asn Ser Leu Glu Pro Glu Pro Trp Phe Phe Lys
        115                 120                 125
Asn Leu Ser Arg Lys Asp Ala Glu Arg Gln Leu Leu Ala Pro Gly Asn
    130                 135                 140
Thr His Gly Ser Phe Leu Ile Arg Glu Ser Glu Ser Thr Ala Gly Ser
145                 150                 155                 160
Phe Ser Leu Ser Val Arg Asp Phe Asp Gln Asn Gln Gly Glu Val Val
                165                 170                 175
Lys His Tyr Lys Ile Arg Asn Leu Asp Asn Gly Gly Phe Tyr Ile Ser
            180                 185                 190
Pro Arg Ile Thr Phe Pro Gly Leu His Glu Leu Val Arg His Tyr Thr
        195                 200                 205
Asn Ala Ser Asp Gly Leu Cys Thr Arg Leu Ser Arg Pro Cys Gln Thr
    210                 215                 220
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(ix) FEATURE:

(A) NAME/KEY: Other
(B) LOCATION: 6...6
(D) OTHER INFORMATION: Xaa = Phosphorylated Tyrosine

(A) NAME/KEY: Other
(B) LOCATION: 17...17
(D) OTHER INFORMATION: Xaa = Phosphorylated Tyrosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Gly Ser Asn Gln Leu Xaa Asn Glu Leu Asn Leu Gly Arg Arg Glu Glu
1 5 10 15
Xaa Asp Val Leu Asp Lys
20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GATCGCCATG GGAGTGCAGG TGGAAACCAT CTCCCCA 37

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TACGAATTCT GGCGTGGATC CACGCGGAAC CAGACCTTCC AGTTTTAG 48

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATTAGGATCC ATGCCAGATC CTGCAGCTCA CCTGCCCT 38

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATATGGATCC TTACCAGAGG CGTTGCT 27

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CAATAGGATC CATGGCCAGC AGCGGCATGG CTGA 34

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GACCTAGGAT CCCTAATTAA CATTTCCCTG TGTGCCGAT 39

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATATGGATCC ATGGCGAACA GCCTGGAGCC CGAACCCT 38

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ATTAGGATCC TTAGGTCTGG CAGGGGCGGC TCAACCGTGT GCA 43

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(ix) FEATURE:

(A) NAME/KEY: Other
(B) LOCATION: 4...4
(D) OTHER INFORMATION: XAA = PHOSPHORYLATED TYROSINE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Glu Pro Gln Xaa Glu Glu Ile Pro Ile Tyr Leu
1 5 10

WHAT IS CLAIMED IS:

1. A method of screening for compounds capable of binding to a fusion protein which comprises the steps of:

a) mixing a test compound, a biotinylated ligand, the fusion protein, a donor-labeled ligand and acceptor-labeled streptavidin;

b) incubating the mixture for a suitable time period;

c) measuring the time-resolved fluorescence attributable to the binding of the biotinylated ligand to the fusion protein in the presence of the test compound; and

d) determining the binding of the biotinylated ligand to the fusion protein in the presence of the test compound relative to a control assay run in the absence of the test compound.

2. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 1, wherein the acceptor-labeled streptavidin consists of streptavidin coupled to a light harvesting molecule.

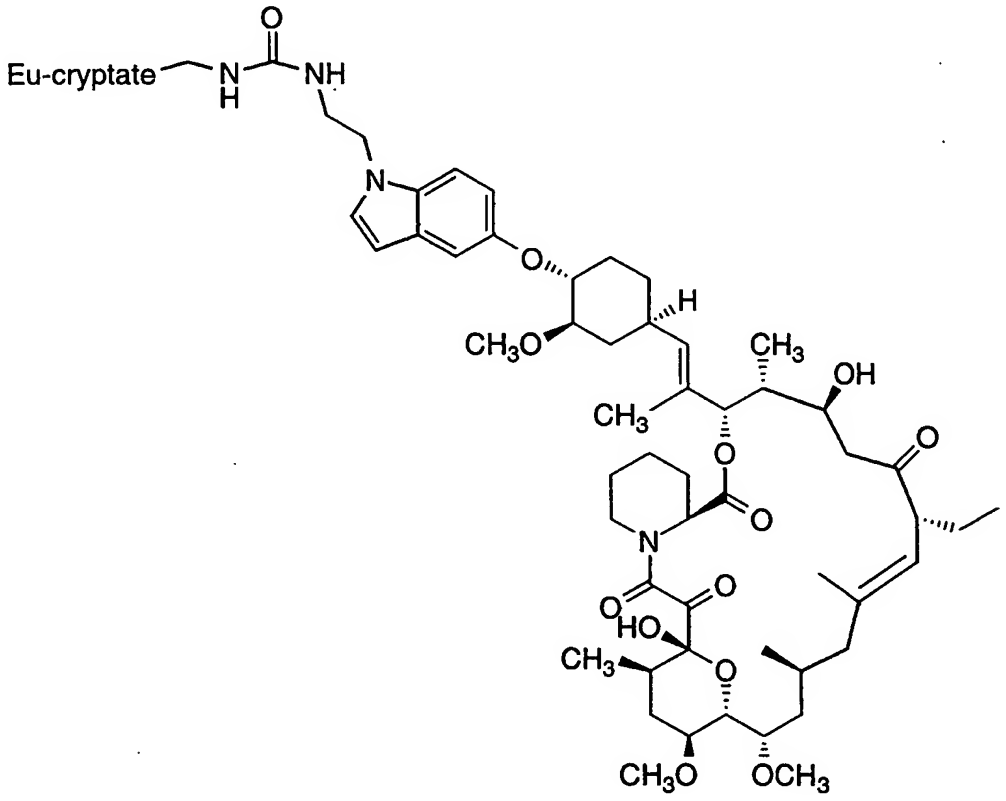
3. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 2, wherein the acceptor-labeled streptavidin consists of SA-XL665.

4. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 3, wherein the donor-labeled ligand comprises a cryptate-containing molecule coupled to a lanthanide metal.

5. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 4, wherein the donor-labeled ligand is selected from the group consisting of a Eu(K)-labeled analog of FK506 or and Eu(K)-labeled analog of rapamycin.

6. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 5, wherein the donor-labeled ligand is an Eu(K)-labeled analog of FK506.

7. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 6, wherein the donor-labeled ligand is



8. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 7, wherein the fusion protein comprises an FK506 binding protein, peptide linker and target protein.

9. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 8, wherein the FK506 binding protein is 12kDA human FK506 binding protein.

10. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 9, wherein the target protein is selected from tyrosine phosphatases, nuclear receptors, bacterial tRNA synthetases or single and multiple signal transduction domains.

11. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 10, wherein the target protein comprises a single or multiple signal transduction domain.

12. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 11, wherein the single or multiple signal transduction domain is selected from the group consisting of: SH1, SH2, SH3 and PH domains.

13. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 12, wherein the target protein is a single or multiple SH2 domain.

14. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 13, wherein the target protein is a single or multiple SH2 domain selected from the group consisting of: ZAP70:SH2, Syk:SH2 and Lck:SH2.

15. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 14, wherein the target protein is the SH2 domain, ZAP70:SH2.

16. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 14, wherein the target protein is the SH2 domain, Syk:SH2.

17. The method of screening for compounds capable of binding to a fusion protein, as recited in Claim 14, wherein the target protein is the SH2 domain, Lck:SH2.

18. A method of screening for compounds capable of inhibiting a protease which comprises the steps of:

a) mixing a test compound, a fusion protein substrate, a donor-labeled ligand, acceptor-labeled streptavidin, and a protease;

b) incubating the mixture for an appropriate time;

c) measuring the time-resolved fluorescence attributable to the binding of the intact fusion protein substrate to the acceptor-labeled streptavidin in the presence of the test compound; and

d) determining the binding of the intact fusion protein substrate to the acceptor-labeled streptavidin in the presence of the test compound relative to a control assay run in the absence of the test compound.

19. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 18, wherein the protease is selected from the group consisting of thrombin, HIV or TNFα converting enzyme.

20. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 19, wherein the protease is thrombin.

21. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 20, wherein the acceptor-labeled streptavidin consists of streptavidin coupled to a light harvesting molecule.

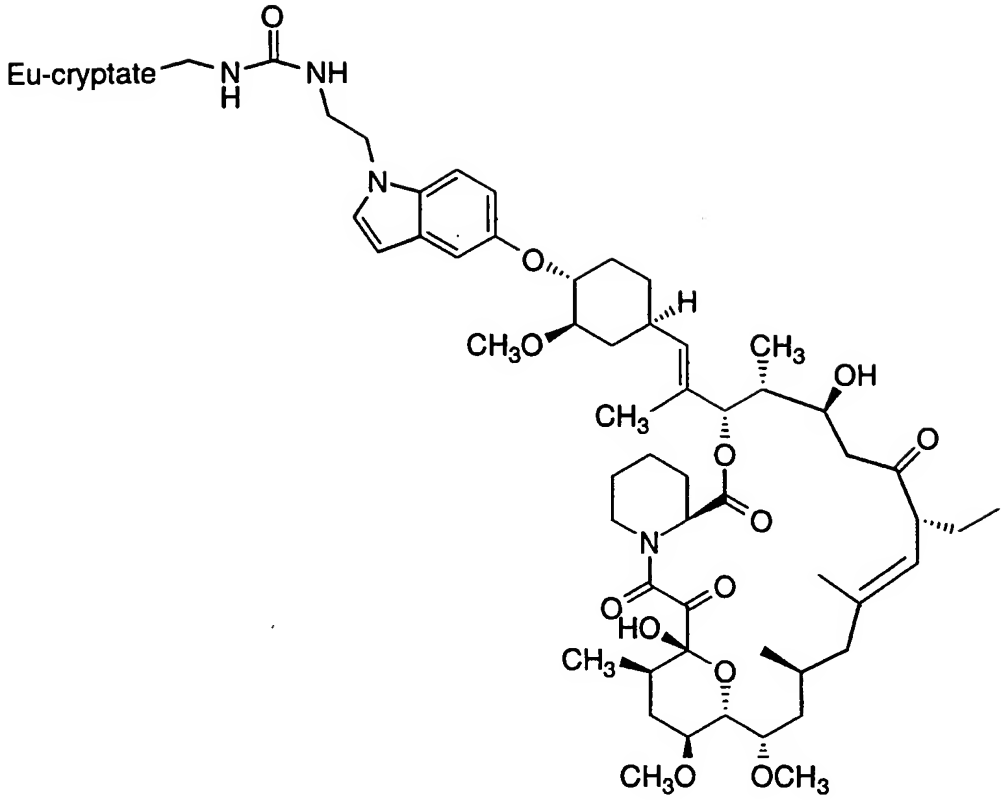
22. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 21, wherein the acceptor-labeled streptavidin consists of SA-XL665.

23. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 22, wherein the donor-labeled ligand comprises a cryptate-containing molecule coupled to a lanthanide metal.

24. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 23, wherein the donor-labeled ligand is selected from the group consisting of an Eu(K)-labeled analog of FK506 or and Eu(K)-labeled analog of rapamycin.

25. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 24, wherein the donor-labeled ligand is an Eu(K)-labeled analog of FK506.

26. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 25, wherein the donor-labeled ligand is



27. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 26, wherein the fusion protein comprises an FK506 binding protein, peptide linker and reporter protein.

28. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 27, wherein the FK506 binding protein is 12kDA human FK506 binding protein.

29. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 28, wherein the reporter protein is selected from the group consisting of SH2 domains or covalently biotinylated proteins.

30. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 29, wherein the covalently biotinylated protein is selected from the group consisting of the C-terminal 87 residues of the biotin carboxy carrier protein of acetyl-CoA carboxylase from *Escherichia coli* or the biotin-carrier subunit of transcarboxylase from *Propionibacterium shermannii.*

31. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 29, wherein the SH2 domain is the ZAP70 bound to a biotinylated phosphopeptide derived from an ITAM sequence.

32. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 31, wherein the ITAM sequence is selected from the group consisting of the human T-cell receptor, the B-cell receptor or a high affinity IgE receptor.

33. The method of screening for compounds capable of inhibiting a protease, as recited in Claim 32, wherein the ITAM sequence is the Zeta 1 sequence of the human T-cell receptor. (SEQ. ID. NO. 8)

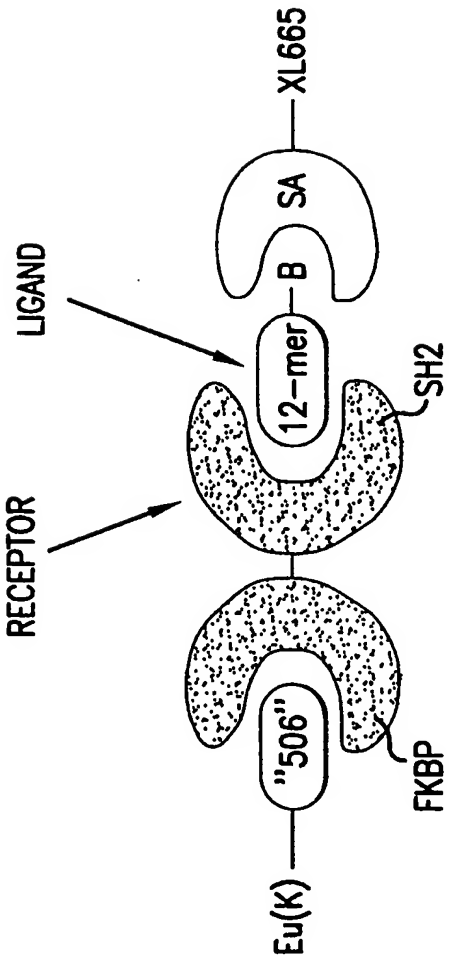


FIG.1

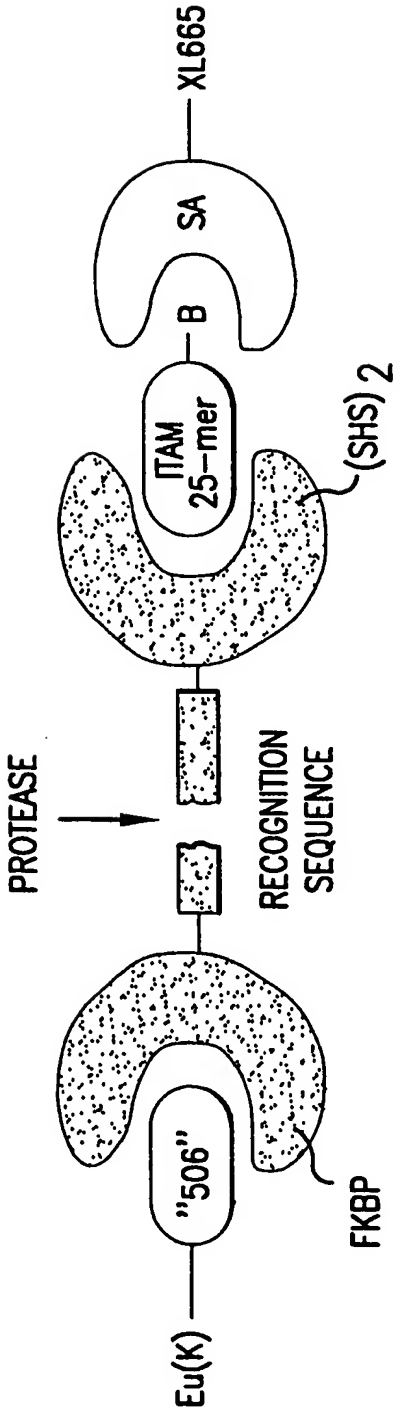

XL665
SA
B
ITAM
25-mer
(SHS)2
PROTEASE
RECOGNITION
SEQUENCE
"506"
FKBP
Eu(K)

FIG.2

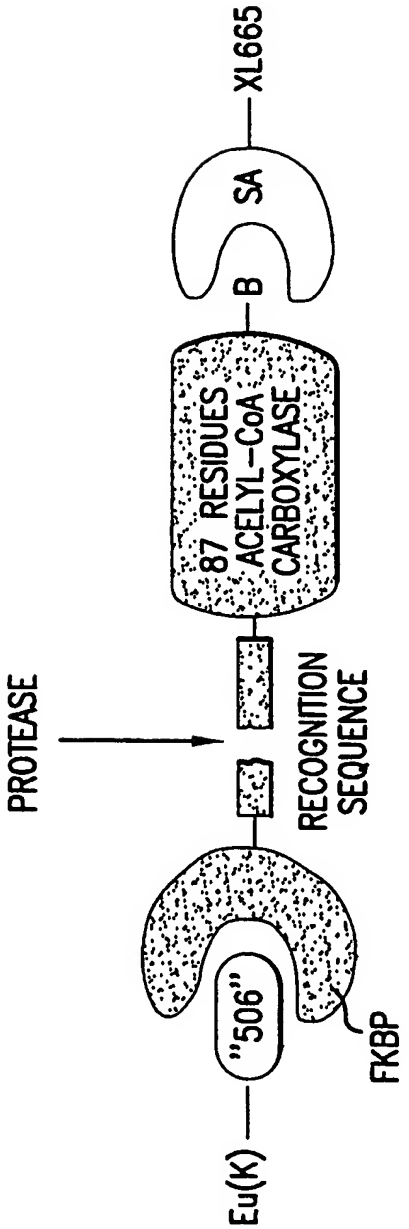

XL665
SA
B
87 RESIDUES
ACELYL-CoA
CARBOXYLASE
PROTEASE
RECOGNITION
SEQUENCE
"506"
FKBP
Eu(K)

FIG.3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/04610

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/53, 33/544, 33/531
US CL :435/7.1, 7.5; 436/529, 543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 7.5; 436/543

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, GENBANK, BIOTECHDS, BIOSIS, CA, MEDLINE, LIFESCI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 97/10502 A1 (MERCK & CO., INC.) 20 March 1997, see entire document.	1-33
A	US 5,498,597 A (BURAKOFF et al) 12 March 1996, see entire document.	1-33
A	US 5,352,660 A (PAWSON) 04 October 1994, see entire document.	1-33

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 21 MAY 1998	Date of mailing of the international search report 16 JUL 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JENNIFER SHAVER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★